Journal of Clinical Microbiology, Jan. 2005, p. 57–65 Vol. 43, No. 1
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.1.57–65.2005

Assessing the Serodiagnostic Potential of 35 *Mycobacterium tuberculosis* Proteins and Identification of Four Novel Serological Antigens

Karin Weldingh,* Ida Rosenkrands, Limei Meng Okkels, T. Mark Doherty, and Peter Andersen

Department of Infectious Disease Immunology, Statens Serum Institut, Copenhagen, Denmark

Received 21 July 2004/Accepted 5 September 2004

Improved diagnostic reagents are needed for the detection of *Mycobacterium tuberculosis* infections, and the development of a serodiagnostic test would complement presently available diagnostic methods. The aim of the present study was to identify novel serological targets for use for the future serodiagnosis of tuberculosis (TB). We cloned and expressed 35 *M. tuberculosis* proteins as recombinant proteins in *Escherichia coli* and analyzed their serodiagnostic potentials. By a two-step selection process, four superior seroantigens, TB9.7, TB15.3, TB16.3, and TB51, were identified, none of which has been described before. The four novel antigens were tested with panels of sera from smear-positive and smear-negative TB patients from areas both where TB is endemic and where TB is not endemic, with recognition frequencies ranging from 31 to 93% and with a specificity of at least 97%. The single most potent antigen was TB16.3, which had a sensitivity of 48 to 55% with samples from Danish resident TB patients and a sensitivity of 88 to 98% with samples from African TB patients. Importantly, the TB16.3 and the TB9.7 antigens were recognized by more than 85% of the samples from TB patients coinfected with human immunodeficiency virus, a patient group for which it is in general difficult to detect *M. tuberculosis*-specific antibodies.

Approximately one-third of the world's population is infected with *Mycobacterium tuberculosis*, and 7 million to 8 million new cases of tuberculosis (TB) occur each year (49). The majority of TB cases occur in developing countries with limited resources, and today, the diagnosis of TB largely depends upon clinical examination and radiographic findings, confirmed by sputum smear microscopy and bacterial culture. Culture is not useful as a first-line means of diagnosis due to the long cultivation period required for *M. tuberculosis* (6 to 8 weeks); therefore, confirmation of the diagnosis relies on sputum smear examinations. However, smear microscopy has a sensitivity of only 50 to 60%, and the detection rate in children and patients coinfected with human immunodeficiency virus (HIV) is even lower (for a review, see reference 41).

To improve the diagnosis of TB, more rapid diagnostic techniques have been investigated in recent years. These methods include the detection of *M. tuberculosis* components in clinical specimens by PCR and immune reactions, based on the cell-mediated immune (CMI) response or on humoral immune responses. The in vitro CMI-based tests allow the early detection of latently infected individuals and are of tremendous value for contact tracing and screening of high-risk groups in a setting of low endemicity (10, 16). However, this method is not suitable as an alternative to culture and microscopy in developing countries, as large proportions of the populations in these countries are likely to be latently infected with *M. tuberculosis* (24).

A serological test is an attractive diagnostic method because it is rapid, easy to perform, and robust and can easily be implemented under the conditions commonly encountered in developing countries, as it does not require living cells, whereas the CMI-based diagnostic assays do. In recent years considerable progress has been made in the identification of serological antigens, and several promising seroantigens have been identified. The 38-kDa antigen has been the most frequently studied and has been included in a number of studies. The sensitivities of assays with this antigen have been reported to range from 16 to 80%, depending on the smear status of the patient and the patient populations used in the studies (3, 27, 42; for a review, see reference 20). This antigen is also included as a component in three different commercial immunochromatographic test kits (ICT Tuberculosis AMRAD-ICT [Amrad, Sydney, Australia], RAPID test TB, and PATHOZYME-MYCO), which detect TB in from 25 to 64% of patients with smear-negative and smear-positive TB (33, 34). Other antigens tested as targets for serology-based tests include α-crystallin (HspX), MTB48, and Mtb81 (21, 26, 27). Finally, ESAT6 and CFP10, two antigens that have been used for CMI-based tests, have also been evaluated as targets of humoral immune responses (14, 27, 42).

No commercially available serological test has so far shown useful levels of sensitivity and specificity, which may be due to the great heterogeneity of the antibody response in TB patients (27), and it is therefore generally accepted that it will be necessary to include several antigens in a future serodiagnostic assay. Through the use of both cocktails of single proteins and genetically engineered fusion molecules containing several antigens, it has been demonstrated that the necessary improvements in sensitivity can be achieved by combining the best antigens (17, 22).

The aim of this study was therefore to identify additional immunodominant serological antigens. For this purpose we cloned and screened 35 recombinantly produced *M. tuberculosis* proteins and identified four strongly recognized antigens which have not previously been described as serological tar-

* Corresponding author: Mailing address: Department of Infectious Disease Immunology, Statens Serum Institut, Artillerivej 5, DK-2300 Copenhagen S, Denmark. Phone: 45 32683817. Fax: 45 32683035. E-mail: kwe@ssi.dk.

TABLE 1. Primers used for PCR amplification and cloning

Rv no. of gene[i]	Forward primer sequence (5′ → 3′)[a]	Reverse primer sequence (3′ → 5′)[a]
Rv0164	CTG AGA TCT CCA GTT TTG AGC AAG ACC[b]	CTC CCA TGG GCA CAT GCC TTA GCT GGC[c]
Rv0285	CTG AGA TCT ATG ACG TTG CGA GTG GTT	CTC CCA TGG TCA GCC GCC CAC GAC CCC[c]
Rv0577	CTGA AGA TCT ATG CCC AAG AGA AGC GAA TAC[b]	CGG CAG CTG CTA GCA TTC TCC GAA TCT GCC G[d]
Rv0652	CCGGG AGA TCT ATG GCA AAG CTC TCC ACC GAC G[b]	CGTA CCA TGG GAG CTA CTT GAC GGT GAC GGT[c]
Rv0984	CCC AAG CTT ATG GAA CAG CGT GCG GAG[e]	CTC CCA TGG CGA CAC TCG ATC CGG ATT[e]
Rv1335	CTG AGA TCT ATG AAC GTC ACC GTA TCC[b]	TCT GGG CCC GCT CAC CCA CCG GCC ACG[f]
Rv1636	CTG AAG CTT ATG AGC GCC TAT AAG ACC[e]	CTG CCA TGG CTA GGT GGT GTG CAC GAT C[c]
Rv1827	AC AGA TCT GTG ACG GAC ATG AAC CCG[b]	TTTT CCA TGG TCA CGG GCC CCC GGT ACT[c]
Rv1932	AC AGA TCT GTG CCC ATG GCA CAG ATA[b]	TTT AAG CTT CTA GGC GCC CAG CGC GGC[e]
Rv1984c	AC AGA TCT GCG CAT GCG GAT CCG TGT[c]	TTTT CCA TGG TCA TCC GGC GTG ATC GAG[c]
Rv2110c	CTGCCG AGA TCT ACC ACC ATT GTC GCG CTG AAA TAC CC[b]	CG CCA TGG CCT TAC GCG CCA ACT CG[b]
Rv2140c	GAGGA AGA TCT ATG ACA ACT TCA CCC GAC CCG[b]	CATGAAG CCA TGG CCC GCA GGC TGC ATG[c]
Rv2185c	CTG AGA TCT GCG GAC AAG ACG ACA CAG[b]	CTC CCA TGG TAC CGG AAT CAC TCA GCC[c]
Rv2244	CTG AGA TCT CCT GTC ACT CAG GAA GAA[b]	CTC CCA TGG GAA ACC GCC ATT AGC GGT[c]
Rv2462c	CTG AGA TCT GTG AAG AGC ACC GTC GAG[b]	CTC CCA TGG GTC ATA CGG TCA CGT TGT[c]
Rv2623	CTG AGA TCT ATG TCA TCG GGC AAT TCA[b]	CTC CCA TGG CTAC CTA AGT CAG CGA CTC GCG[c]
Rv2716	GGCCC AGA TCT ATG GCC ATT GAG GTT TCG GTG TTG C[b]	CGC CGT GTT CCA TGG CAG CGC TGA GC[c]
Rv3354	CTG AGA TCT ATG AAC CTA CGG CGC C[b]	CTC CCA TGG TAC CCT AGG ACC CGG GCA GCC CCG GC[c]
Rv3451	CCTTGGG AGA TCT TTG GAC CCC GGT TGC[b]	GACG AGA TCT TAT GGG CTT ACT GAC[b]
Rv3592	CTG AGA TCT ATG CCA GTG GTG AAG ATC[b]	CTC CCA TGG TTA TGC AGT CTT GCC GGT[c]
Rv3803	CTC GAA TTC CGC GGG GCC GGG TGC ACA CAG[g]	GAG GAA TTC GCT TAG CGG ATC GCA[g]
Rv3891	CTG AGA TCT ATG GCA GAC ACA ATT CAGG[b]	AAG CTT CCC GGG TCA GGA TCC GTG GCT AGC[h]
TB9.4	CTG AGA TCT GTG GAG GTC AAG ATC GGT[b]	CTC CCA TGG CTAC TTA CCC GCT CGT AGC AAC[c]

[a] The restriction sites used for cloning are underlined.
[b] BglII.
[c] NcoI.
[d] PvuII.
[e] HindIII.
[f] ApaI.
[g] Eco RI.
[h] SmaI.
[i] According to the nomenclature of Cole et al. (12).

gets. One of these, TB16.3, was the single most frequently recognized antigen in patients both from regions where TB is endemic and regions where TB is not endemic, as well as from TB patients coinfected with HIV.

MATERIALS AND METHODS

Cloning of *M. tuberculosis* open reading frames into *Escherichia coli* expression vectors. The cloning of ESAT-6, TB7.3, TB9.9, CFP10, TB10.4, CFP22, CFP25, TB27.4, CFP29, TB37.6, and MPT64 has been described previously (1, 9, 19, 30, 32, 37, 43, 47).

The gene-specific primers used for cloning of the other open reading frames included in the present study are listed in Table 1. The PCRs were conducted in a 50-μl mixture with Taq^+ DNA polymerase (Stratagene, La Jolla, Calif.) and 10 ng of *M. tuberculosis* H37Rv DNA. The primer concentrations were 0.4 μM, and the nucleotide concentrations were 250 μM. The PCRs were initiated by a denaturation step at 94°C for 2 min, followed by 30 cycles of denaturation (94°C, 30 s), annealing (55°C, 30 s), and extension (72°C, 90 s) and, subsequently, a final extension step (72°C, 10 min). The PCR products were cloned into N-terminal His-tagged expression vector pMCT3 at the restriction sites indicated in Table 1 and transformed into XL-1 Blue cells. Mpt51 was cloned into the pMAL-*cR1* expression vector (New England Biolabs, Beverly, Mass.).

Recombinant protein expression and purification. Recombinant antigens ESAT-6, TB7.3, TB9.9, CFP10, TB10.4, CFP22, CFP25, TB27.4, CFP29, TB37.6, and MPT64 were purified as His-tagged proteins as described previously (1, 2, 30, 32, 43). The recombinant 38-kDa antigen was kindly provided by M. Singh. Recombinant antigen TB9.7 was purified as follows: *E. coli* cells expressing high levels of rTB9.7 were lysed by sonication in 20 mM Tris-HCl (pH 8.0)–0.5 M NaCl–8 M urea–10% glycerol (buffer A). The supernatant from this mixture was loaded on a Talon column (Clontech, Palo Alta, Calif.), and the bound proteins were eluted stepwise with buffer A containing from 5 to 100 mM imidazole. Fractions containing TB9.7 were pooled and dialyzed against buffer A containing

1 mM imidazole and were then purified through a second Talon column. Finally, rTB9.7 was further purified on a Hitrap Q column (Amersham Biosciences, Hillerød, Denmark) in 10 mM Tris-HCl (pH 8.5)–3 M urea–10% glycerol–0.01% Tween 20–1 mM EDTA with a linear gradient of 0 to 1 M NaCl. Recombinant TB51 was purified in a similar way, except that the second Talon column contained 50 mM NaH_2PO_4–10 mM Tris-HCl–8 M urea–100 mM NaCl (pH 8.0) and the bound proteins were eluted by pH steps, as described in the manual of the manufacturer (Clontech).

MPT51 was produced as a fusion with the maltose binding protein and was purified by affinity chromatography on an amylose resin column eluted with 10 mM maltose in a manner similar to that described by Oettinger et al. (31). The remaining recombinant proteins included in this study were produced as described above for TB9.7, but they were purified through only one Talon column before proceeding to the Hitrap Q column. The purified recombinant proteins were stored in 25 mM HEPES (pH 7.5)–0.15 M NaCl–10% glycerol–0.01% Tween 20 at −20°C.

The protein concentration was determined by the bicinchoninic acid method of Pierce Chemical Company (Rockford, Ill.).

Study population. Sera were collected from four different groups of individuals. (i) Sera were collected from 119 HIV-negative TB patients (92 males and 27 females) recruited at Danish hospitals. Forty-nine were born in Denmark, 31 were born in Asia, 23 were born in southeast Africa, and 16 were born in other parts of the world. Ninety-seven of these patients had pulmonary TB, and 92 of these were culture positive for *M. tuberculosis*. One patient was culture negative and smear microscopy positive; and four patients were culture and microscopy negative. The diagnosis of TB for these five patients was based on X-ray and/or clinical findings and/or patient history. Twenty-two patients had extrapulmonary TB (12 lymph node, 6 pleural cavity, 1 intestinal, 1 paratracheal, 1 peritoneal, and 1 psoas abscess). Thirty-one patients (27 with pulmonary TB and 4 with extrapulmonary TB) were smear microscopy negative. Smear microscopy was not performed for 23 patients (10 with extrapulmonary TB and 13 with pulmonary TB). The mean age of the patients in this group was 43 years.

For the initial screening 18 serum samples (from 16 males and 2 females, 15 of whom had pulmonary TB and 3 of whom had extrapulmonary TB) were selected on the basis of the presence of high levels of antimycobacterial antibodies in the serum sample, as determined by an enzyme-linked immunosorbent assay (ELISA) with *M. tuberculosis* short-term culture filtrate (ST-CF). A panel of 48 randomly selected TB patients (34 males and 14 females, 37 of whom had pulmonary TB and 11 of whom had extrapulmonary TB) was used as the derivation panel (and for the establishment of the ELISA cutoff value). Ten patients were smear microscopy negative, and smear microscopy was not performed for 12 patients. The 71 patients not included in the derivation panel (58 males and 13 females, 60 of whom had pulmonary TB and 11 of whom had extrapulmonary TB) were tested as part of the validation panel. Twenty-one patients were smear negative, and smear microscopy was not performed for 11 patients. For the calculation of sensitivity with samples from patients with extrapulmonary TB, samples from all 22 patients recruited at Danish hospitals and diagnosed with extrapulmonary TB were included, regardless of the smear microscopy result. Samples from all 27 patients with smear-negative pulmonary TB were used for calculation of the sensitivity of detection of smear-negative pulmonary TB.

(ii) The second group, which comprised healthy volunteers living in Denmark ($n = 69$; 36 males and 33 females) with no known exposure to *M. tuberculosis*, was used as a negative control group. Thirty-five individuals had not been vaccinated with *M. bovis* BCG, and 34 had been vaccinated with BCG more than 15 years earlier. The mean age of the volunteers in this group was 41 years.

For the initial screening, six healthy non-BCG-vaccinated controls (three males and three females) were used. The derivation panel included 32 healthy controls (17 males and 15 females). Fifteen of these had been vaccinated with BCG, and 17 had not. Twenty-five of these healthy controls were used as internal controls in all experiments.

The validation panel included the remaining 31 healthy controls (16 males and 15 females). Nineteen of these had been vaccinated with BCG, and 12 had not.

(iii) The third group comprised HIV-negative pulmonary TB patients living in Uganda ($n = 72$; 55 males and 17 females). All were culture and smear microscopy positive. The mean age of the patients in this group was 28 years.

(iv) The fourth group comprised HIV-positive TB patients living in Uganda ($n = 79$; 48 males and 31 females). All were culture and smear microscopy positive. The mean age of the patients in this group was 32 years.

The serum samples from Uganda were obtained through the World Health Organization Tropical Disease Research Tuberculosis Specimen bank.

The study was approved by the Local Ethical Committee for Copenhagen and Frederiksberg (approvals RH 01-282/96 and KF 01-369/98), and written consent forms were obtained from the patients.

Antibody detection by ELISA. The antigen coating concentration of 1 μg/ml was establish by testing three antigens (the 38-kDa, CFP21, and TB16.3 antigens) at concentrations ranging from 5 to 0.1 μg/ml. The optimal serum dilution of 1:100 was established by titrating 15 serum samples from 1:100 to 1:12,800 on ST-CF-coated plates. All samples were run in duplicates. The ELISA procedure was performed as follows: 96-well polystyrene microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 μl of antigen solution (1 μg/ml for single antigens and 3 μg/ml for ST-CF) in 0.05 M carbonate buffer (pH 9.6). After the plates were coated, they were washed three times with phosphate-buffered saline (PBS; pH 7.2)–0.05% Tween 20 (PBS-T), and serum samples diluted 1:100 in PBS were applied and incubated for 1 h at room temperature. After the plates were washed with PBS-T, they were incubated for 1 h with rabbit anti-human immunoglobulin G antibody conjugated with horseradish peroxidase (D0336; Dako, Hillerød, Denmark) diluted 1:1,000 in PBS-T. The plates were washed with PBS-T, and enzyme activity was assayed by incubation for 30 min at room temperature with 100 μl of tetramethylbenzidine peroxidase substrate (Bio-Rad, Hercules, Calif.) per well. To stop the reaction, 100 μl of 4 N sulfuric acid was added, and the optical density (OD) was measured at 405 nm.

Data analysis. The ELISA results were analyzed by using cutoff values equal to the mean OD for the healthy control serum samples plus 3 standard deviations.

To allow comparison between groups and to control for interplate as well as day-to-day variations, the absorbance readings were transformed into arbitrary units by defining the cutoff as 1 on the basis of the mean OD of 25 for the defined healthy control serum samples plus 3 standard deviations. If the variation for these serum samples was more than 15%, the data were discarded.

For statistical analysis, the difference between groups of TB patients and healthy controls was calculated by the nonparametric Mann-Whitney rank sum test by use of the SigmaStat software package (SPSS Inc., Chicago, Ill.). Differences were considered statistically significant if the P value was <0.05.

RESULTS

Selection of *M. tuberculosis* proteins recognized serologically by sera of TB patients. We have cloned and produced 35 *M. tuberculosis* proteins in *E. coli*, all of which have previously been identified by proteomic or genomic approaches (Table 2). Most of the proteins have been demonstrated to be expressed in *M. tuberculosis* either in vivo or in vitro, and several of the proteins were selected due to their recognition by T cells after an *M. tuberculosis* infection. With a few exceptions (the 38-kDa antigen and the ESAT6, CFP10, MPT64, and MPT51 antigens), the antibody responses of TB patients to these proteins have not been investigated previously.

For the first screening step we attempted to identify a broad panel of targets by using sera from 18 selected TB patients who all had high titers of antibody toward ST-CF, which is a complex mixture of proteins from *M. tuberculosis*. The presence of specific antibodies in these blood samples was compared to the presence of antibodies in healthy controls, who were all negative for a response to ST-CF. In general, the antibody responses to the antigens could be divided into three different categories, as illustrated in Fig. 1. For most of the antigens (category 1) the antibody levels in the sera of TB patients were higher than those in the sera of the healthy controls. This should be compared to the category 2 antigens, to which the sera from both the healthy controls and the patients had high antibody levels. Finally, the third category of antigens was characterized by low antibody responses by the sera from patients as well as the sera from the controls. Antigens in categories 2 and 3 were not selected for further analysis, and although category 1 clearly contained antigens with diagnostic potential, for several of the antigens only a small difference between the signal in patients and that in the controls was detected. We therefore applied a selection criterion based on the difference between the mean for the responding patients

TABLE 2. Serological screening of 35 recombinantly produced *M. tuberculosis* proteins

Protein name	Rv no. of gene[f]	Mol mass (kDa)	Reference(s)	Response catagory[a]	ΔOD value[b]	Selection[c]
TB7.3[d]	Rv3221c	7.3	36	1	0.21	+
TB9.4	Rv3208A	9.4	11, 36	3	0.11	−
TB9.56	Rv0285	9.57	45	3	0.1	−
TB9.6	Rv1335	9.6	23	1	0.17	−
TB9.7	Rv3354	9.7	NP[e]	1	0.20	+
ESAT-6	Rv3875	9.9	44	1	0.18	−
TB9.9	Rv3872	9.9	7	1	0.27	+
TB10.4	Rv0288	10.4	36	3	0.1	−
CFP10	Rv3874	10.8	9	1	0.20	+
TB11.16	Rv3891	11.2	45	1	0.18	−
TB11.2	Rv3592	11.2	36	1	0.18	−
AcpM	Rv2244	12.5	36	3	0.14	−
TB13.4	Rv0652	13.4	36	1	0.29	+
TB15.3	Rv1636	15.3	36	1	0.27	+
TB16.3	Rv2185c	16.3	36	1	0.23	+
MoaB2	Rv0984	16.8	36	3	0.12	−
CFP20	Rv1932	16.9	47	2	0.1	−
CFP17	Rv1827	17.2	47	1	0.27	+
TB18.5	Rv0164	18.5	36	1	0.19	−
TB18.6	Rv2140c	18.6	46	1	0.15	−
CFP21	Rv1984c	18.9	47	1	0.32	+
CFP22	Rv0009	19.2	47	2	−0.70	−
CFP25	Rv2301	19.7	47	1	0.15	−
CFP23	Rv3451	23.2	NP	3	0.12	−
TB24.6	Rv2716	24.6	NP	1	0.15	−
MPT64	Rv1980	24.8	29, 31	1	0.18	−
TB27.3	Rv0577	27.3	36	1	0.16	−
TB27.4	Rv3878	27.4	7	3	0.08	−
MPT51	Rv3803	27.8	29	3	0.12	−
CFP29	Rv0798c	28.9	37	1	0.19	−
PrcB	Rv2110c	30.3	36	2	−0.1	−
TB31.7	Rv2623	31.7	36	2	−0.37	−
38-kDa antigen	Rv0934	35.9	3, 4	1	0.47	+
TB37.6	Rv3873	37.3	7	3	0.13	−
TB51	Rv2462c	50.6	36	1	0.24	+

[a] Antigen response category defined from the antibody response patterns of 18 antibody-positive TB patients and 6 healthy controls.
[b] The change in OD (ΔOD) value is calculated as the mean OD for the control donors subtracted from the mean OD for the TB patient responders.
[c] All antigens with a change in the OD value of > 0.2 were selected.
[d] Boldface indicates the antigens selected for further characterization.
[e] NP, not previously published.
[f] According to the nomenclature of Cole et al. (12).

and the mean for the controls. This allowed selection of the antigens with the best discrimination between TB patients and healthy individuals for use in a diagnostic ELISA. Antigens with a mean OD difference of more than 0.2 were selected (Table 2). Eleven of the antigens, the TB7.3, TB9.7, TB9.9, CFP10, TB13.4, TB15.3, TB16.3, CFP17, CFP21, TB51, and 38-kDa antigens, fulfilled this criterion and were selected for further analysis.

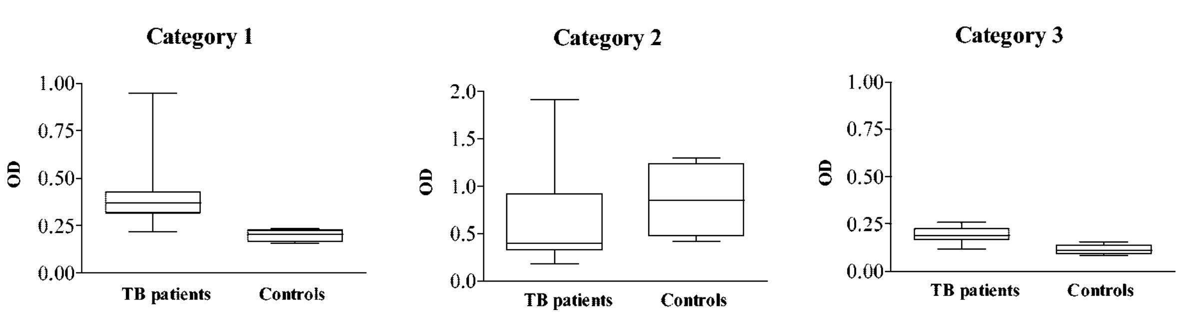

FIG. 1. Box-and-whiskers (25th to 75th percentiles) illustration of the three different types of patterns of antibody levels for 18 selected TB patients with high antibody titers and 6 healthy controls tested with 35 different *M. tuberculosis* antigens. Category 1 represents TB patients with high levels of antibodies and controls with low antibody levels (illustrated with antigen TB16.3), category 2 represents both TB patients and controls with high levels of antibodies (illustrated with antigen CFP22), and category 3 represents both patients and controls with low levels of antibodies (illustrated with antigen TB10.4). The median is shown as a line.

TABLE 3. Sensitivities and specificities of the 11 seroantigens with sera from 48 Danish resident TB patients and 32 Danish healthy controls

Antigen	Cutoff[a]	Sensitivity (%)	Specificity (%)
TB7.3	0.47	10	100
TB9.7	0.37	34	100
TB9.9	0.37	15	100
CFP10	0.40	18	100
TB13.4	0.64	18	97
TB15.3	0.70	31	97
TB16.3	0.49	55	100
CFP17	0.57	13	97
CFP21	0.53	25	97
38-kDa antigen	0.52	19	100
TB51	0.40	31	100

[a] The cutoff was calculated as the mean for the control plus 3 standard deviations.

Establishment of cutoff and selection of the four most potent serological antigens. The 11 antigens were then evaluated with a panel of blood samples collected from 48 TB patients in Denmark, and the antibody levels in the sera from these patients were compared to those in the sera from 31 healthy controls. Individual cutoffs were established for each antigen by use of the mean for the control serum samples plus 3 standard deviations, and the sensitivity and specificity were calculated. For all 11 antigens, the median OD value between the antibody level in the sera from TB patients and the level in the sera from the healthy controls was statistically significantly different ($P < 0.001$). More than 10% of the patient serum samples recognized all antigens, and the most frequently recognized antigen was TB16.3, which was recognized by 55% of the sera from the TB patients tested. All antigens had a specificity of 97% or greater (Table 3).

Between 10 and 20% of the sera from TB patients recognized most of the antigens. However, more than 30% of the sera from the TB patients tested recognized the four antigens TB9.7, TB15.3, TB16.3, and TB51; these superior antigens were therefore selected for further analysis.

Evaluation of the four best antigens with serum panels from areas of high and low endemicities. The frequent presence of TB9.7-, TB15.3-, TB16.3-, and TB51-specific antibodies in TB patients was validated with sera from another panel of Danish resident TB patients, African resident TB patients, and nonexposed healthy controls by using the cutoff established in Table 3, adjusted for day-to-day variance (Fig. 2). The data confirmed that the sensitivities of the antigens selected were from 30 to 50% with sera from the Danish residents, with a specificity of at least 97%. In general, these antigens were more frequently recognized, and the samples collected in Africa had higher antibody titers than the samples collected in Denmark. The most marked finding was that TB9.7 had a sensitivity of 85% with sera from African resident TB patients but a sensitivity of only 36% with sera from Danish resident TB patients.

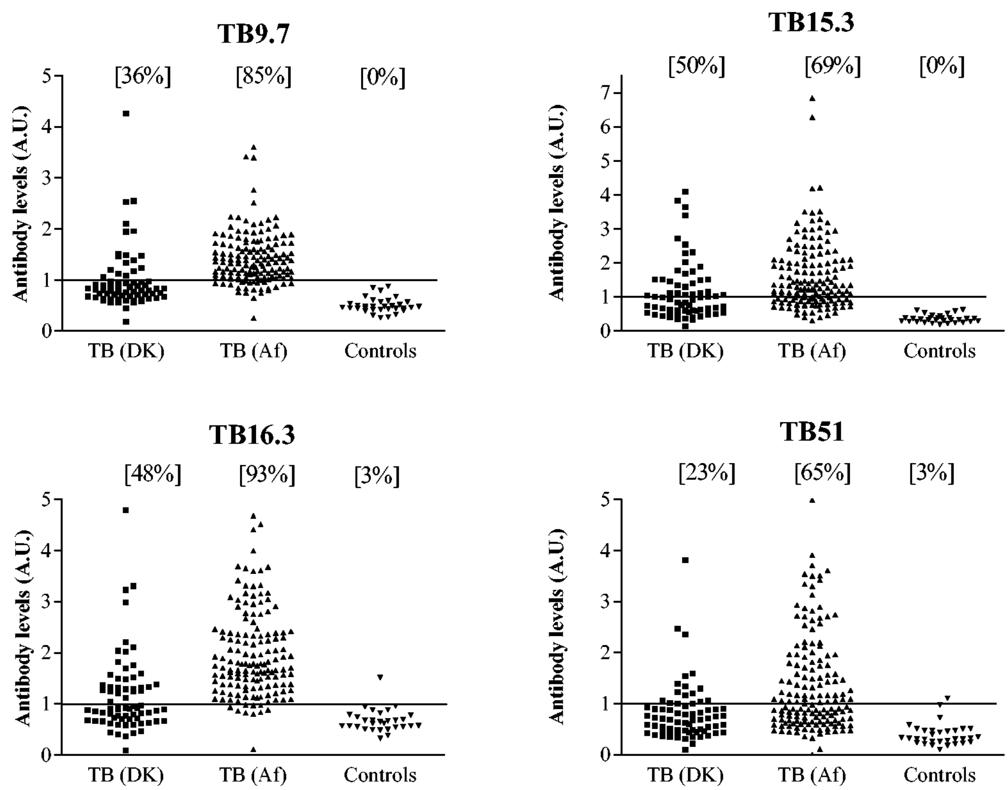

FIG. 2. Normalized levels of antibodies to the four antigens for TB patients resident in Denmark (DK) or Africa (Af) and for healthy controls resident in Denmark with no known exposure to *M. tuberculosis*. Arbitrary units (A.U.) represent the OD for the sample test divided by the cutoff, based on the mean for a defined panel of 25 healthy controls plus 3 standard deviations. The percentage of positive individuals is shown at the top of each column.

TABLE 4. Sensitivities of the four best antigens with samples from smear-negative patients and patients with extrapulmonary TB patients.

Antigen	Sensitivity (%)	
	Extrapulmonary TB patients[a]	Smear-negative pulmonary TB patients[b]
TB9.7	32	37
TB15.3	41	41
TB16.3	46	66
TB51	32	15

[a] Group composed of 22 Danish resident TB patients with extrapulmonary TB.
[b] Group composed of 27 Danish resident TB patients with smear-negative pulmonary TB.

More than 65% of the serum samples from African patients recognized all four antigens, and TB16.3 was the single most frequently recognized antigen by the sera from both patient populations, with recognition frequencies of 93% for sera from African patients and 48% for sera from Danish resident patients. In comparison, antibodies specific for the 38-kDa antigen were found in sera from 22% of the Danish TB patients and 28% of the African population (data not shown).

Serological performance of the four antigens with samples from smear-negative and extrapulmonary TB patients. By present diagnostic methods, pulmonary TB and smear-positive TB are often easily recognized, whereas the diagnosis of extrapulmonary TB and smear-negative TB is more difficult. It would therefore be an advance if a novel diagnostic tool could be used with samples from these patient groups. We evaluated the potential of the four serological antigens to identify patients with smear-negative pulmonary TB or extrapulmonary TB (Table 4). For all antigens the sensitivities with samples from patients with extrapulmonary TB and smear-negative TB were more than 32%, and sensitivities of 41 to 68% were obtained with TB15.3 and TB16.3 with samples from these patient groups. However, only 15% of the samples from patients with pulmonary smear-negative TB recognized TB51.

TB9.7 is frequently recognized by TB patients coinfected with HIV. A large proportion of TB patients are coinfected with HIV, and it has been reported that HIV-positive patients generally have lower levels of *M. tuberculosis*-specific antibodies than HIV-negative patients (13). The sensitivities obtained with TB16.3, TB9.7, TB15.3, and TB51 were therefore compared with samples from HIV-negative and HIV-positive TB patients (Table 5). All four antigens were also frequently recognized by samples (>50%) from this population; however, they were recognized at a level lower than that observed for the HIV-negative patients. One exception was TB9.7, the sensitivity of which was slightly higher for the patient group coinfected with HIV. Although the difference was not statistically significant ($P = 0.110$), this result shows that the generally suppressed antibody response in HIV-positive patients does not preclude the strong recognition of some antigens, and TB16.3 and TB9.7 gave sensitivities as high as 88 and 91% with samples from this patient population.

TABLE 5. Sensitivities of the four best antigens with samples from HIV-negative and HIV-positive TB patients

Antigen	Sensitivity (%) by the following HIV infection status:	
	Negative[a]	Positive[b]
TB9.7	79	91
TB15.3	75	65
TB16.3	98	88
TB51	82	50

[a] Group composed of 72 African resident TB patients.
[b] Group composed of 79 African resident TB patients coinfected with HIV.

DISCUSSION

In this study we used the ELISA technique to evaluate the serological responses to a panel of 35 recombinantly produced antigens from *M. tuberculosis*. After a stepwise selection process we identified four antigens, TB9.7, TB15.3, TB16.3, and TB51, that have serodiagnostic potential; none of these has been described before. Sera from different groups of patients frequently recognized these antigens; and the TB16.3 antigen was the single most important antigen, with levels of recognition of 46 to 98% and maintenance of a specificity of 97%. The TB9.7 antigen was particularly effective for the serodiagnosis of TB-HIV coinfections and detected TB in 91% of these patients, and the combination of TB9.7 and TB16.3 detected TB in 96% of the HIV-positive patients (data not shown). In our study the performances of these four novel antigens surpassed that of the 38-kDa antigen, which was recognized by 19 to 28% of the sera from the different patient subpopulations. The 38-kDa antigen is the most frequently studied serological antigen and is also a core component in several different commercial TB serological tests (ICT Tuberculosis AMRAD-ICT, RAPID test TB, and PATHOZYME-MYCO). The recognition frequency reported for the 38-kDa antigen varies tremendously (from 16 to 94%), largely depending on the smear status and disease manifestation. The first reports of the use of antibodies specific for the 38-kDa antigen in TB patients for the diagnosis of TB were done either by using purified native 38-kDa antigen (also called antigen 5 or antigen 78) or by competition ELISA with an *M. tuberculosis* extract and specific monoclonal antibodies (for a review, see reference 20). By using the native purified antigen, 68 to 94% sensitivity and >91% specificity were obtained by direct ELISA analysis (5, 8, 15). By a competition ELISA with soluble *M. tuberculosis* extract-coated plates and monoclonal antibodies specific for the 38-kDa antigen for the detection of competing antibodies in human sera, 85% of smear-positive pulmonary cases, 74% of cases of extrapulmonary TB, and 70% of smear-negative cases were detected (48). However, in this study two of the five controls (40%) were also positive. Both of these relatively small studies are based on the recognition of the 38-kDa antigen in its native form, either purified or present in a protein mixture; and recognition of this antigen may be different from that of the recombinant version of the antigen used in later work, including the present study. It is well described that the native 38-kDa antigen is a lipoglycoprotein, and similar posttranslational modification would not be expected for the recombinant antigen produced in *E. coli* (50). In this regard, Samanich et al. (39) have reported differential humoral recognition of recombinantly produced reagents and native antigens, most likely as a consequence of the lack of posttranslational modification and structural differences in the recombinant proteins. Furthermore, it is possible that antigens purified from a complex *M. tuberculosis* mixture may be contaminated with trace amounts of other *M. tuberculosis* proteins and may thereby lead to false-positive responses. Gamma interferon responses to native purified and recombinantly produced GroES were found to be markedly different; and in one study it was concluded that the differences observed could be due to the presence of trace amounts of other *M. tuberculosis* protein components copurified with GroES, even though one- and two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the purified GroES protein showed no visible signs of contamination (38). It is therefore very likely that part of the explanation for the different performances reported with the 38-kDa antigen may reflect differences in the source of the antigen used. Later studies have reported lower sensitivities with the recombinant version of the 38-kDa antigen, with sensitivities by the ELISA technique ranging from 16 to 36% for smear-negative patients and from 36 to 67% for smear-positive patients (14, 26, 27, 42). Moreover, it is clear that there is also a significant variance in the results obtained by using the same antigen with samples from different populations, as observed, for example, by Houghton et al. (22), who found that the frequency of recognition by use of the same recombinant 38-kDa antigen preparation ranged from 35 to 82% with samples from smear-positive TB patients from four different geographic areas. In our study we also observed a large variation in sensitivity when we tested the same antigens with sera from TB patients living in Denmark and sera from TB patients living in Uganda; and in accordance with earlier results (20), we found that the highest proportion of positive antibody responses was by serum samples from regions where TB is highly endemic. In addition to the differences in ethnic backgrounds, it is likely that there is a difference in the stage of disease at which the patients from areas of high and low endemicity are admitted to the hospital, and this parameter may also influence the magnitude of the antibody response mounted by the patients.

During recent years, antigens with performances similar to or better than that of the 38-kDa protein have been identified. In this study we have identified a panel of potentially promising, useful reagents. The increased pace of discovery programs is due to improved methods, such as screening of expression libraries (22, 26, 28) or (in our study) the systematic screening of recombinant antigens. As discussed above, there may be a marked difference in the performances of native and recombinant antigens in serodiagnostic assays, and until recently, discovery programs started out with the identification of target molecules in native protein preparations, followed by recombinant expression and evaluation of the antigen. In this regard we have applied a reverse discovery program in which antigens have been evaluated and selected on the basis of their performance as recombinant antigens. Most of the proteins included in the present study have, however, also been mapped in proteome studies of subcellular fractions of *M. tuberculosis* (36) (data not shown). One of the promising seroantigens, TB9.7, has not been described previously, and this protein was identified by N-terminal sequencing of protein bands in *M. tuberculosis* ST-CF fractions in a manner similar to that described for antigen CFP21 (47). Analysis of the protein sequence of TB9.7 in databases reveals no putative function of this protein; but it contains a typical secretion signal, and sequencing of the N terminus of native material showed cleavage of the signal peptide from the mature proteins (data not shown). This protein would therefore be expected to be present outside the bacterium, and in accordance with this, TB9.7 is found in the culture filtrate (36). TB15.3, TB16.3, and TB51 are all present in cell wall preparations of *M. tuberculosis* proteins and may therefore also be present on the outside of the mycobacterium. Putative functions have been assigned only to TB51, which has homology to chaperone proteins and which is assigned to the functional class involved in cell wall and cell processes, according to the nomenclature of Cole et al. (12).

It is striking that the best serological antigens identified in this study are all antigens that are secreted or present in the cell wall of *M. tuberculosis*. Other secreted or cell wall-associated proteins which are described as good serological targets are the 38-kDa antigen, members of the antigen 85 (Ag85) complex, and MPT32 (22, 39, 40); and one of three novel seroantigens identified by Houghton et al. (22), Mtb8 (Rv0379), is most likely a cell wall-associated transporter protein. As the presence of *M. tuberculosis*-specific circulating antibodies does not play a role in the outcome of the disease, the parameters needed for the development of an antibody response have not yet been defined. However, even though *M. tuberculosis* is an intracellular bacterium and is therefore protected from the actions of antibodies, it is possible that the cellular localization of the antigen may influence the accessibility and, subsequently, the production of antibodies specific for *M. tuberculosis* antigens. Interestingly, cell wall- or membrane-associated proteins have been identified as immunodominant B-cell targets in other intracellular bacterial species, such as *Chlamydia*, *Listeria*, and *Ehrlichia* (6, 18, 25).

The advantage of using an antibody-based assay instead of CMI-based techniques is that a serological assay can be developed into a simple and robust kit format ideal for use in a field setting in, for example, a developing country. In this study we have used sera from TB patients from regions of both high and low endemicity to identify serological targets that are frequently recognized by sera from both patients groups. It is now generally recognized that latently infected people are positive by CMI-based assays that use ESAT-6 and CFP10 as the antigens (24, 35). Whether these people also have antibodies specific for *M. tuberculosis* antigens is not known, but investigation of this parameter is important, as this will markedly influence the performance of a serological test in an area where TB is endemic. Therefore, a study of the response to the selected antigens in community controls from an area where TB is endemic is an important study that must be performed.

We have identified four potent serological antigens which, when used in combination, had a very high predicted sensitivity for the detection of TB in 75% of the Danish donors and 97% of the African donors (data not shown). It is apparent that attempts to fuse these antigens into a hybrid molecule should be made and that a single molecule should be produced in a way similar to that described previously (22). This would simplify the production and greatly reduce the cost of a future serology test kit, which is an important concern if the test is to be viable in the developing world. Of concern with the use of this strategy is whether the conformational B-cell epitopes will be conserved in such a hybrid molecule. This will need to be thoroughly examined by using the single proteins for comparison.

ACKNOWLEDGMENTS

We thank the WHO/TDR Tuberculosis Specimen bank for providing the serum samples collected in Uganda. Axel Kok-Jensen, Inger Brock, and Pernille Ravn are thanked for their efforts with the collection of serum samples in Denmark, and Patricia Grenés and Jette Fløjborg are thanked for their valuable technical assistance. M. Singh is thanked for providing the 38-kDa antigen for this study.

Karin Weldingh, Ida Rosenkrands, and Peter Andersen are the named inventors on patents relating to the discovery of the proteins described in this report. All rights have been assigned to the Statens Serum Institut. None of the authors has any financial interest in the study.

The study was supported by the Danish National Association against Lung Disease.

REFERENCES

1. **Aagaard, C., M. Govaerts, L. Meng Okkels, P. Andersen, and J. M. Pollock.** 2003. Genomic approach to identification of *Mycobacterium bovis* diagnostic antigens in cattle. J. Clin. Microbiol. **41:**3719–3728.
2. **Agger, E. M., I. Brock, L. M. Okkels, S. M. Arend, C. S. Aagaard, K. N. Weldingh, and P. Andersen.** 2003. Human T-cell responses to the RD1-encoded protein TB27.4 (Rv3878) from Mycobacterium tuberculosis. Immunology **110:**507–512.
3. **Andersen, A. B., and E. B. Hansen.** 1989. Structure and mapping of antigenic domains of protein antigen b, a 38,000-molecular-weight protein of *Mycobacterium tuberculosis*. Infect. Immun. **57:**2481–2488.
4. **Andersen, A. B., A. Worsaae, and S. D. Chaparas.** 1988. Isolation and characterization of recombinant lambda gt11 bacteriophages expressing eight different mycobacterial antigens of potential immunological relevance. Infect. Immun. **56:**1344–1351.
5. **Balestrino, E. A., T. M. Daniel, M. D. de Latini, O. A. Latini, Y. Ma, and J. B. Scocozza.** 1984. Serodiagnosis of pulmonary tuberculosis in Argentina by enzyme-linked immunosorbent assay (ELISA) of IgG antibody to Mycobacterium tuberculosis antigen 5 and tuberculin purified protein derivative. Bull. W. H. O. **62:**755–761.
6. **Bannantine, J. P., R. S. Griffiths, W. Viratyosin, W. J. Brown, and D. D. Rockey.** 2000. A secondary structure motif predictive of protein localization to the chlamydial inclusion membrane. Cell Microbiol. **2:**35–47.
7. **Behr, M. A., M. A. Wilson, W. P. Gill, H. Salamon, G. K. Schoolnik, S. Rane, and P. M. Small.** 1999. Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science **284:**1520–1523.
8. **Benjamin, R. G., and T. M. Daniel.** 1982. Serodiagnosis of tuberculosis using the enzyme-linked immunoabsorbent assay (ELISA) of antibody to Mycobacterium tuberculosis antigen 5. Am. Rev. Respir. Dis. **126:**1013–1016.
9. **Berthet, F. X., P. B. Rasmussen, I. Rosenkrands, P. Andersen, and B. Gicquel.** 1998. A Mycobacterium tuberculosis operon encoding ESAT-6 and a novel low-molecular-mass culture filtrate protein (CFP-10). Microbiology **144**(Pt 11)**:**3195–3203.
10. **Brock, I., M. E. Munk, A. Kok-Jensen, and P. Andersen.** 2001. Performance of whole blood IFN-gamma test for tuberculosis diagnosis based on PPD or the specific antigens ESAT-6 and CFP-10. Int. J. Tuberc. Lung Dis. **5:**462–467.
11. **Camus, J. C., M. J. Pryor, C. Medigue, and S. T. Cole.** 2002. Re-annotation of the genome sequence of Mycobacterium tuberculosis H37Rv. Microbiology **148:**2967–2973.
12. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry III, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, B. G. Barrell, et al.** 1998. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature **393:**537–544.
13. **Daniel, T. M., A. A. Sippola, A. Okwera, S. Kabengera, E. Hatanga, T. Aisu, S. Nyole, F. Byekwaso, M. Vjecha, L. E. Ferguson, et al.** 1994. Reduced sensitivity of tuberculosis serodiagnosis in patients with AIDS in Uganda. Tuber. Lung Dis. **75:**33–37.
14. **Dillon, D. C., M. R. Alderson, C. H. Day, T. Bement, A. Campos-Neto, Y. A. Skeiky, T. Vedvick, R. Badaro, S. G. Reed, and R. Houghton.** 2000. Molecular and immunological characterization of *Mycobacterium tuberculosis* CFP-10, an immunodiagnostic antigen missing in *Mycobacterium bovis* BCG. J. Clin. Microbiol. **38:**3285–3290.
15. **Espitia, C., I. Cervera, R. Gonzalez, and R. Mancilla.** 1989. A 38-kD Mycobacterium tuberculosis antigen associated with infection. Its isolation and serologic evaluation. Clin. Exp. Immunol. **77:**373–377.
16. **Ewer, K., J. Deeks, L. Alvarez, G. Bryant, S. Waller, P. Andersen, P. Monk, and A. Lalvani.** 2003. Comparison of T-cell-based assay with tuberculin skin test for diagnosis of Mycobacterium tuberculosis infection in a school tuberculosis outbreak. Lancet **361:**1168–1173.
17. **Gennaro, M. L.** 2000. Immunologic diagnosis of tuberculosis. Clin. Infect. Dis. **30**(Suppl. 3)**:**S243–S246.
18. **Grenningloh, R., A. Darji, J. Wehland, T. Chakraborty, and S. Weiss.** 1997. Listeriolysin and IrpA are major protein targets of the human humoral response against *Listeria monocytogenes*. Infect. Immun. **65:**3976–3980.
19. **Harboe, M., A. S. Malin, H. S. Dockrell, H. G. Wiker, G. Ulvund, A. Holm, M. C. Jorgensen, and P. Andersen.** 1998. B-cell epitopes and quantification of the ESAT-6 protein of *Mycobacterium tuberculosis*. Infect. Immun. **66:**717–723.
20. **Harboe, M., and H. G. Wiker.** 1992. The 38-kDa protein of Mycobacterium tuberculosis: a review. J. Infect. Dis. **166:**874–884.
21. **Hendrickson, R. C., J. F. Douglass, L. D. Reynolds, P. D. McNeill, D. Carter, S. G. Reed, and R. L. Houghton.** 2000. Mass spectrometric identification of mtb81, a novel serological marker for tuberculosis. J. Clin. Microbiol. **38:**2354–2361.
22. **Houghton, R. L., M. J. Lodes, D. C. Dillon, L. D. Reynolds, C. H. Day, P. D. McNeill, R. C. Hendrickson, Y. A. Skeiky, D. P. Sampaio, R. Badaro, K. P. Lyashchenko, and S. G. Reed.** 2002. Use of multiepitope polyproteins in serodiagnosis of active tuberculosis. Clin. Diagn. Lab. Immunol. **9:**883–891.
23. **Jungblut, P. R., U. E. Schaible, H. J. Mollenkopf, U. Zimny-Arndt, B. Raupach, J. Mattow, P. Halada, S. Lamer, K. Hagens, and S. H. Kaufmann.** 1999. Comparative proteome analysis of Mycobacterium tuberculosis and Mycobacterium bovis BCG strains: towards functional genomics of microbial pathogens. Mol. Microbiol. **33:**1103–1117.
24. **Lalvani, A., P. Nagvenkar, Z. Udwadia, A. A. Pathan, K. A. Wilkinson, J. S. Shastri, K. Ewer, A. V. Hill, A. Mehta, and C. Rodrigues.** 2001. Enumeration of T cells specific for RD1-encoded antigens suggests a high prevalence of latent Mycobacterium tuberculosis infection in healthy urban Indians. J. Infect. Dis. **183:**469–477.
25. **Li, J. S., and G. M. Winslow.** 2003. Survival, replication, and antibody susceptibility of *Ehrlichia chaffeensis* outside of host cells. Infect. Immun. **71:**4229–4237.
26. **Lodes, M. J., D. C. Dillon, R. Mohamath, C. H. Day, D. R. Benson, L. D. Reynolds, P. McNeill, D. P. Sampaio, Y. A. Skeiky, R. Badaro, D. H. Persing, S. G. Reed, and R. L. Houghton.** 2001. Serological expression cloning and immunological evaluation of MTB48, a novel *Mycobacterium tuberculosis* antigen. J. Clin. Microbiol. **39:**2485–2493.
27. **Lyashchenko, K., R. Colangeli, M. Houde, H. Al-Jahdali, D. Menzies, and M. L. Gennaro.** 1998 Heterogeneous antibody responses in tuberculosis. Infect. Immun. **66:**3936–3940.
28. **Manca, C., K. Lyashchenko, R. Colangeli, and M. L. Gennaro.** 1997. MTC28, a novel 28-kilodalton proline-rich secreted antigen specific for the *Mycobacterium tuberculosis* complex. Infect. Immun. **65:**4951–4957.
29. **Nagai, S., H. G. Wiker, M. Harboe, and M. Kinomoto.** 1991. Isolation and partial characterization of major protein antigens in the culture fluid of *Mycobacterium tuberculosis*. Infect. Immun. **59:**372–382.
30. **Oettinger, T., and A. B. Andersen.** 1994. Cloning and B-cell-epitope mapping of MPT64 from *Mycobacterium tuberculosis* H37Rv. Infect. Immun. **62:**2058–2064.
31. **Oettinger, T., A. Holm, I. M. Mtoni, A. B. Andersen, and K. Hasloov.** 1995. Mapping of the delayed-type hypersensitivity-inducing epitope of secreted protein MPT64 from *Mycobacterium tuberculosis*. Infect. Immun. **63:**4613–4618.
32. **Okkels, L. M., I. Brock, F. Follmann, E. M. Agger, S. M. Arend, T. H. Ottenhoff, F. Oftung, I. Rosenkrands, and P. Andersen.** 2003. PPE protein (Rv3873) from DNA segment RD1 of *Mycobacterium tuberculosis*: strong recognition of both specific T-cell epitopes and epitopes conserved within the PPE family. Infect. Immun. **71:**6116–6123.
33. **Perkins, M. D., M. B. Conde, M. Martins, and A. L. Kritski.** 2003. Serologic diagnosis of tuberculosis using a simple commercial multiantigen assay. Chest **123:**107–112.
34. **Pottumarthy, S., V. C. Wells, and A. J. Morris.** 2000. A comparison of seven tests for serological diagnosis of tuberculosis. J. Clin. Microbiol. **38:**2227–2231.
35. **Ravn, P., A. Demissie, T. Eguale, H. Wondwosson, D. Lein, H. A. Amoudy, A. S. Mustafa, A. K. Jensen, A. Holm, I. Rosenkrands, F. Oftung, J. Olobo, F. von Reyn, and P. Andersen.** 1999. Human T cell responses to the ESAT-6 antigen from Mycobacterium tuberculosis. J. Infect. Dis. **179:**637–645.
36. **Rosenkrands, I., A. King, K. Weldingh, M. Moniatte, E. Moertz, and P. Andersen.** 2000. Towards the proteome of *Mycobacterium tuberculosis*. Electrophoresis **21:**3740–3756.
37. **Rosenkrands, I., P. B. Rasmussen, M. Carnio, S. Jacobsen, M. Theisen, and P. Andersen.** 1998. Identification and characterization of a 29-kilodalton protein from *Mycobacterium tuberculosis* culture filtrate recognized by mouse memory effector cells. Infect. Immun. **66:**2728–2735.
38. **Rosenkrands, I., K. Weldingh, P. Ravn, L. Brandt, P. Hojrup, P. B. Rasmussen, A. R. Coates, M. Singh, P. Mascagni, and P. Andersen.** 1999. Differential T-cell recognition of native and recombinant *Mycobacterium tuberculosis* GroES. Infect. Immun. **67:**5552–5558.
39. **Samanich, K., J. T. Belisle, and S. Laal.** 2001. Homogeneity of antibody responses in tuberculosis patients. Infect. Immun. **69:**4600–4609.

40. **Samanich, K. M., M. A. Keen, V. D. Vissa, J. D. Harder, J. S. Spencer, J. T. Belisle, S. Zolla-Pazner, and S. Laal.** 2000. Serodiagnostic potential of culture filtrate antigens of *Mycobacterium tuberculosis*. Clin. Diagn. Lab. Immunol. **7:**662–668.
41. **Siddiqi, K., M. L. Lambert, and J. Walley.** 2003. Clinical diagnosis of smear-negative pulmonary tuberculosis in low-income countries: the current evidence. Lancet Infect. Dis. **3:**288–296.
42. **Silva, V. M., G. Kanaujia, M. L. Gennaro, and D. Menzies.** 2003. Factors associated with humoral response to ESAT-6, 38 kDa and 14 kDa in patients with a spectrum of tuberculosis. Int. J. Tuberc. Lung Dis. **7:**478–484.
43. **Skjot, R. L., T. Oettinger, I. Rosenkrands, P. Ravn, I. Brock, S. Jacobsen, and P. Andersen.** 2000. Comparative evaluation of low-molecular-mass proteins from *Mycobacterium tuberculosis* identifies members of the ESAT-6 family as immunodominant T-cell antigens. Infect. Immun. **68:**214–220.
44. **Sorensen, A. L., S. Nagai, G. Houen, P. Andersen, and A. B. Andersen.** 1995. Purification and characterization of a low-molecular-mass T-cell antigen secreted by *Mycobacterium tuberculosis*. Infect. Immun. **63:**1710–1717.
45. **Tekaia, F., S. V. Gordon, T. Garnier, R. Brosch, B. G. Barrell, and S. T. Cole.** 1999. Analysis of the proteome of Mycobacterium tuberculosis in silico. Tuber. Lung Dis. **79:**329–342.
46. **Weldingh, K., A. Hansen, S. Jacobsen, and P. Andersen.** 2000. High resolution electroelution of polyacrylamide gels for the purification of single proteins from Mycobacterium tuberculosis culture filtrate. Scand. J. Immunol. **51:**79–86.
47. **Weldingh, K., I. Rosenkrands, S. Jacobsen, P. B. Rasmussen, M. J. Elhay, and P. Andersen.** 1998. Two-dimensional electrophoresis for analysis of *Mycobacterium tuberculosis* culture filtrate and purification and characterization of six novel proteins. Infect. Immun. **66:**3492–3500.
48. **Wilkins, E. G., and J. Ivanyi.** 1990. Potential value of serology for diagnosis of extrapulmonary tuberculosis. Lancet **336:**641–644.
49. **World Health Organization.** 2003. Global tuberculosis controls, p. 1–49. World Health Organization, Geneva, Switzerland.
50. **Young, D. B., and T. R. Garbe.** 1991. Lipoprotein antigens of Mycobacterium tuberculosis. Res. Microbiol. **142:**55–65.